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NOTES

¹Editor's note: SEER is a set of geographically defined, population-based, central cancer registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Registry data are submitted electronically without personal identifiers to the NCI on a biannual basis, and the NCI makes the data available to the public for scientific research.

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$$\textcircled{1} - 7 \times 7 \times 7 \times 0.5 \approx 200 \text{ mM}^3$$

DT-Diaphorase Expression and Tumor Cell Sensitivity to 17-Allylamino,17-demethoxygeldanamycin, an Inhibitor of Heat Shock Protein 90

Lloyd R. Kelland, Swee Y. Sharp, Paul M. Rogers, Timothy G. Myers, Paul Workman

$\textcircled{4}$ 2 human CC lines

Background: To our knowledge, 17-allylamino,17-demethoxygeldanamycin (17AAG) is the first inhibitor of heat shock protein 90 (Hsp90) to enter a phase I clinical trial in cancer. Inhibition of Hsp90, a chaperone protein (a protein that helps other proteins avoid misfolding pathways that produce inactive or aggregated states), leads to depletion of important oncogenic proteins, including Raf-1 and mutant p53 (also known as TP53). Given its ansamycin benzoquinone structure, we questioned whether the antitumor activity of 17AAG was affected by expression of the NQO1 gene, which encodes the quinone-metabolizing enzyme DT-diaphorase. **Methods:** The antitumor activity of 17AAG and other Hsp90 inhibitors was determined by use of a sulforhodamine B-based cell growth inhibition assay in culture and by the arrest of xenograft tumor growth in nude mice. DT-diaphorase activity was determined by use of a spectrophotometric assay, and protein expression was determined by means of western immunoblotting. **Results:** In two independent *in vitro* human tumor cell panels, we observed a positive relationship between DT-diaphorase expression level and growth inhibition by 17AAG. Stable, high-level expression of the active NQO1 gene transfected into the DT-diaphorase-deficient (by NQO1 mutation) BE human colon carcinoma cell line resulted in a 32-fold increase in 17AAG growth-inhibition activity. Increased sensitivity to 17AAG in the transfected cell line was also confirmed in xenografts. The extent of depletion of Raf-1 and mutant p53 protein con-

firmed that the Hsp90 inhibition mechanism was maintained in cells with high and low levels of DT-diaphorase. 17AAG was shown to be a substrate for purified human DT-diaphorase. **Conclusion:** These results suggest that the antitumor activity and possibly the toxicologic properties of 17AAG in humans may be influenced by the expression of DT-diaphorase. Careful monitoring for NQO1 polymorphism and the level of tumor DT-diaphorase activity is therefore recommended in clinical trials with 17AAG. [*J Natl Cancer Inst* 1999;91:1940-9]

Benzoquinone ansamycins, such as herbimycin and geldanamycin (Fig. 1), exhibit anticancer activity by binding to heat shock protein 90 (Hsp90), a molecular chaperone, and its homologue GRP94 (1,2). In this interaction, geldanamycin competes with adenosine triphosphate at the N-terminal-binding site

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of Hsp90 (3). The interaction results in lysosome-mediated degradation of several important oncogenic proteins, including Raf-1, c-ErbB2, and mutant (but not wild-type) p53 (also known as TP53) (4-6). Clearly, this molecular profile offers considerable potential for antitumor activity. However, both herbimycin and geldanamycin have limitations as drug candidates because of poor stability and hepatotoxicity (7). This has resulted in efforts to discover improved synthetic analogues (8).

One such compound, the 17-allylamino,17-demethoxy analogue of geldanamycin (17-allylamino,17-demethoxygeldanamycin; 17AAG) (Fig. 1), has also been shown to bind to Hsp90 (9). Although, in rodent and dog toxicology studies, 17AAG retains some of geldanamycin's toxicity in the liver, gallbladder, and kidney (10) and National Cancer Institute [NCI] drug data file on 17AAG [11] it has a better therapeutic index. For example, 17AAG exerts antitumor activity against some human melanoma xenografts at nontoxic doses [NCI drug data file on 17AAG and (11)]. Preclinical pharmacokinetic studies show that pharmacologically active concentrations can be achieved in plasma and tissues [NCI drug data file on 17AAG and (12)] and that the major liver microsomal metabolite (shown in Fig. 1) is 17-amino,17-demethoxygeldanamycin (13). In view of its novel mechanism of action and its good therapeutic index, 17AAG has now entered phase I clinical trials as first-in-class Hsp90 inhibitor under the auspices of the U.S. NCI and the U.K. Cancer Research Campaign (CRC). Recently, the structurally distinct macrocyclic antifungal compound radicicol (Fig. 1) has been shown to bind to Hsp90 and inhibit its activity (14-17).

DT-diaphorase, an obligate two-electron-reducing enzyme [reduced nicotinamide-adenine dinucleotide (phosphate):quinone oxidoreductase; EC 1.6.99.2], catalyzes the reduction of various quinones (18). As a result, cells rich in DT-diaphorase are especially sensitive to quinone-containing bioreductive anticancer agents, such as mitomycin C and the indoloquinone EO9, which act as prodrugs for activation to toxic forms by DT-diaphorase (19-21). Some tumor types (notably, colon and non-small-cell lung cancers) have been shown to contain rela-

tively high levels of DT-diaphorase (22-26). Thus, these cancers may be particularly suitable for treatments that use a DT-diaphorase prodrug approach. Although previous studies (27) have shown that geldanamycin is a substrate for DT-diaphorase, a cell line derived from human colorectal cancer and expressing DT-diaphorase did not appear to be particularly sensitive to geldanamycin. However, it is not known whether cells expressing high levels of DT-diaphorase show altered sensitivity to 17AAG.

The primary aim of this study was to investigate whether DT-diaphorase activity has a role in the sensitivity of human tumor cells to 17AAG. Initially, sensitivity to 17AAG was determined by use of the CRC/Institute of Cancer Research (ICR) panel of 15 human colorectal and 11 ovarian carcinoma cell lines, including some resistant to classical agents. Comparative data were obtained in selected lines for the 17-amino metabolite and the additional Hsp90-binding agents geldanamycin and radicicol. The correlation between sensitivity and DT-diaphorase activity seen in a subset of the CRC/ICR panel (selected to span the range of sensitivity to 17AAG) was then examined and confirmed with data from the NCI panel of 60 human tumor cell lines (28). This led to the hypothesis that high DT-diaphorase expression was a major factor in determining cellular sensitivity to 17AAG but not to geldanamycin or radicicol. To provide further conclusive data, sensitivity to 17AAG was determined in a newly established isogenic pair of cell lines that differ only in the expression of the active NQO1 gene. This pair is composed of the human colon BE line [which contains a disabling point mutation in the NQO1 gene encoding DT-diaphorase (29)] and a subline stably transfected with the NQO1 gene and expressing high levels of functional DT-diaphorase. Finally, evidence that the Hsp90 inhibitory mechanism was retained by 17AAG in colon cell lines expressing high and low levels of DT-diaphorase was obtained by immunoblot analysis of Raf-1, mutant p53, Hsp70, and Hsp90 proteins. The results suggest that determination of patients' NQO1 genotype and of tumor DT-diaphorase activity should be included in the clinical evaluation of 17AAG because variations in these characteristics could affect the toxicity and efficacy of the drug.

MATERIALS AND METHODS

Cell Lines

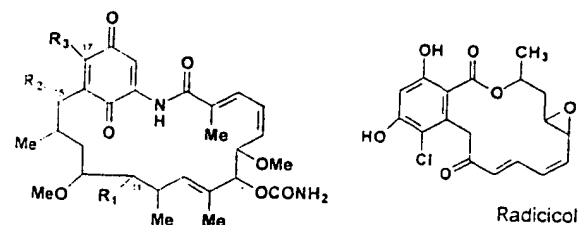
We used panels of human colon and ovarian cell lines. We obtained cell lines from commercial cell culture collections or derived them in-house as described previously (30). In some cases, we used sublines derived from a particular parent line with acquired drug resistance to cisplatin (CH1cisR and A2780cisR ovarian lines) or to doxorubicin (CH1doxR and an SKOV-3 subline stably overexpressing the multidrug-resistance protein MRP1) (30-32). All lines were grown as monolayers in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 2 mM glutamine, and 0.5 µg/mL hydrocortisone in 6% CO₂/94% air. All lines were free of *Mycoplasma* contamination.

Drugs and Chemicals

Geldanamycin, 17AAG, and 17-amino,17-demethoxygeldanamycin were supplied by E. Sausville (NCI). The remaining drugs (herbimycin, radicicol, streptonigrin, and dicoumarol) and chemicals were obtained from Sigma Chemical Co. (Poole, U.K.).

Growth Inhibition Studies

We used the sulforhodamine B assay as described previously (30-32) for growth inhibition studies. Briefly, we seeded tumor cells into 96-well microtiter plates, allowed the cells to attach overnight, and then added the drug to quadru-



	R ₁	R ₂	R ₃
Geldanamycin	OH	H	OMe
17AAG	OH	H	CH ₂ =CHCH ₂ NH
17-amino	OH	H	NH ₂
Herbimycin	OMe	OMe	H

Fig. 1. Chemical structures of geldanamycin, 17-allylamino,17-demethoxygeldanamycin (17AAG), 17-amino,17-demethoxygeldanamycin (17-amino), radicicol, and herbimycin.

uplicate wells as indicated. Unless otherwise indicated, we exposed cells to a drug for 4 days. Thereafter, the cell number in treated versus control wells was estimated after treatment with 10% trichloroacetic acid and staining with 0.4% sulforhodamine B in 1% acetic acid. The IC_{50} was calculated as the drug concentration that inhibits cell growth by 50% compared with control growth.

Stable Transfection of the NQO1 Gene Into the BE Human Colon Carcinoma Cell Line

BE cells contain a point mutation in the NQO1 gene and thus have no functional DT-diaphorase enzyme activity (29). We used the bicistronic expression vector pEFIRE5-P (33) to express the NQO1 gene in BE cells. Lipofectamine (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) for transfection, and puromycin (0.5 μ g/mL) for selection. Resulting clones were screened for DT-diaphorase enzyme activity or protein by an enzyme assay or immunoblotting, respectively (see below). Full details of the vector construction and the biologic properties of the stable transfectants will be published elsewhere (Sharp SY, Kelland LR, Valenti MR, Branton LA, Hobbs S, Workman P; unpublished results). The stable transfectants, designated BE-F397 clone 2 and BE-F397 clone 5, were used in these studies.

DT-Diaphorase Assay

To determine whether 17AAG was a good substrate for DT-diaphorase, we used the standard cytochrome c assay, as described previously for the bioreductive indoloquinone EO9 (34) and geldanamycin (27), but replaced menadione with 17AAG as the substrate and intermediate electron acceptor. We assayed extracts of the human colon cell line HT29 or purified human DT-diaphorase protein (from J. Skelly, ICR). For preparation of cell extracts, 2×10^7 cells were trypsinized, washed twice in ice-cold phosphate-buffered saline (PBS), and centrifuged (MSE Centaur I; 1100 rpm for 5 minutes at room temperature). The cell pellet then was resuspended in 0.5–1 mL of lysis buffer (PBS containing 1% Triton X-114 and 500 μ M phenylmethylsulfonyl fluoride) and left on ice for 30 minutes. After centrifugation (MSE Microcentrifuge; 12000 rpm for 5 minutes at room temperature), the supernatant was used for protein determination and the enzyme assay. Results obtained for 17AAG were compared with those for geldanamycin, EO9, and streptonigrin, an excellent substrate for DT-diaphorase (35). For all drugs, the difference in reduction of the menadione substrate in the absence and presence of dicoumarol (100 μ M), a standard inhibitor of DT-diaphorase, was determined (27).

Immunoblotting

This analysis was performed as described previously (30–32). Briefly, 5×10^6 cells were trypsinized, washed with PBS, and lysed in 100 μ L of lysis buffer at 4 °C for 1 hour. Lysis buffer contained 10 mL of 150 mM NaCl–50 mM Tris–HCl (pH 7.5), 500 μ L of 20 mM phenylmethylsulfonyl fluoride, 2 μ L of aprotinin (10 mg/mL, stock solution), 2 μ L of leupeptin (10 mg/mL, stock solution), 100 μ L of 10 mM sodium orthovanadate, 100 μ L of Nonidet P-40, and 100 μ L of 20% sodium dodecyl sulfate (SDS). Lysates were centrifuged (MSE Microcentrifuge; 12000 rpm for 15 minutes at 4 °C), and the resulting protein extracts were separated (50 μ g/lane) by SDS–polyacrylamide gel electrophoresis and electroblotted to nitrocellulose filters. Antibodies to Hsp90 and Hsp70 were obtained from StressGen (Victoria, Canada), and antibodies to Raf-1 and p53 (DO1) were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody to the rat DT-diaphorase (which cross-reacts with human diaphorase) was supplied by R. Knox (previously at CRC/ICR, now at Enzaeta Ltd., Salisbury, U.K.). Antibody binding was identified with horseradish peroxidase-labeled secondary antibodies combined with enhanced chemiluminescence reagents (Amersham, Buckinghamshire, U.K.) and autoradiography.

In Vivo Effects

BE vector control cells and BE-F397 clone 2 cells were established as subcutaneous xenografts by injection of 5×10^6 cells into the flanks of adult female athymic nude (nu/nu) mice. The antitumor effect of 17AAG was determined in mice bearing comparably sized tumors (6–8 mm in diameter) derived from these cells. Animals were randomly assigned to receive vehicle alone (five or six mice) or 17AAG (five animals; dose schedule = 80 mg/kg per day in 10% dimethyl sulfoxide and 90% egg phospholipid by intraperitoneal injection on days 1–4 and days 7–11). Before this clinical formulation was available, 17AAG was administered to mice bearing HT29 xenografts in 10% dimethyl sulfoxide–0.05% Tween 20–90% NaCl, with a dose schedule of 80 mg/kg per day on days 0–3 and

days 6–10. This dose and schedule were derived from previously performed experiments [NCI drug data file on 17AAG and (11)].

Tumor size was determined twice weekly by caliper measurements, and tumor volumes were calculated (volume = $[a \times b^2 \times \pi]/6$, where a and b are orthogonal tumor diameters). Tumor volumes were then expressed as a percentage of the volume at the start of treatment (relative tumor volume). The effect of the drug was determined by the growth delay, i.e., the difference in days required for the volume of tumors in control and treated animals to double. All procedures involving animals were performed within the guidelines set out by the Institute's Animal Ethics Committee and the United Kingdom Coordinating Committee for Cancer Research's *ad hoc* Committee on the Welfare of Animals in Experimental Neoplasia (36).

Statistical Analyses

Where indicated, errors are presented as standard deviation ($n \geq 3$). Correlation tests and linear regression analyses were computed with SAS JMP (SAS Institute, Cary, NC). We assessed correlations with a Spearman calculation for the CRC/ICR panel and with a Pearson calculation for the NCI panel. Although the Spearman statistic is technically more robust, the Pearson statistic was used for correlations in the NCI panel for historic continuity. The likelihood test for linear model comparison was performed with S-Plus (Mathsoft, Seattle, WA). All P values are two-sided.

RESULTS

In Vitro Growth Inhibition

The *in vitro* growth inhibition properties of geldanamycin, 17AAG, and radicicol against panels of human colon (11 lines) and ovarian (11 lines) carcinoma cell lines are shown in Table 1, A. The IC_{50} value for 17-amino,17-demethoxygeldanamycin, the major metabolite of 17AAG, is also included for some lines. In most cell lines, all four compounds potentially inhibited growth, with IC_{50} values of less than 2.5 μ M. Notably, one ovarian cell line (the 41M line) was relatively resistant ($IC_{50} > 2.5 \mu$ M) to all four Hsp90-interactive compounds. On average, geldanamycin was the most potent agent (mean $IC_{50} = 50.1$ nM), with similar values obtained for 17-amino,17-demethoxygeldanamycin (mean $IC_{50} = 47$ nM in a subset of nine cell lines). 17AAG showed intermediate potency (mean $IC_{50} = 220.4$ nM), and the least potent agent was radicicol (mean $IC_{50} = 587.4$ nM).

Bar graphs showing the IC_{50} values (Fig. 2) reveal some interesting differences in the patterns of response for geldanamycin, 17AAG, and radicicol. Notably, some cell lines (e.g., BE and LoVo colon cells) are relatively resistant to 17AAG but not to geldanamycin (or radicicol). In contrast, the colon cell lines LS174T and KM12 were relatively resistant to geldanamycin but not to 17AAG. We have compared patterns of response for 25 cell lines (excluding 41M because this line was resistant to all compounds) by use of the Spearman analysis. Positive, but not statistically significant, correlations were observed between geldanamycin and radicicol ($r = .36$; $P = .08$) and between geldanamycin and 17AAG ($r = .33$; $P = .11$). There was, however, no correlation between 17AAG and radicicol ($r = -.08$; $P = .72$). Results indicate relatively distinct patterns of response for the three compounds. 17-Amino,17-demethoxygeldanamycin was studied in only a few lines in the panel. With the exception of LS174T colon cells, which are relatively resistant to geldanamycin and more sensitive to the 17-amino metabolite, the two compounds behaved similarly across the panel.

Activity in Acquired Anticancer Drug-Resistant Cell Lines

The *in vitro* potencies of geldanamycin, 17AAG, and radicicol have also been evaluated in various anticancer drug-resistant

Table 1. *In vitro* human tumor cell growth inhibition by 17AAG and other heat shock protein inhibitors

A. Summary of growth inhibition (drug concentrations that inhibit growth by 50% [IC_{50}]) of geldanamycin, 17AAG, radicicol, and 17-amino against the CRC/ICR panel of human colon and ovarian tumor cells*.

IC_{50} , nM

Cell line	Geldanamycin	17AAG	Radicicol	17-Amino
Colon				
BE	19.3 ± 3.1	773 ± 30.6	190	18
HT29	46.7 ± 9	8.9 ± 2.9	3100	6.3 ± 1.8
COLO205	3.8	7.2	1400	ND
DLD-1	78	140	290	ND
HCA-7	1.8	72	120	ND
HCC2998	98	78	650	ND
HCT116	83	490	280	ND
HCT115	67	99	240	ND
HT55	11.3	13.5	390	17
KM12	54	9	135	ND
LIM1215	9	77	100	20.5
LS174T	24.5	78.5	780	33.5
LoVo	21.9	1130	360	42
MAWI	6.2	58	1850	ND
SW620	3.1	68	110	ND
Ovarian				
A2780	11.5	12	115	43
CHI	104.5	1055	325	190
HX62	47	670	2500	ND
IGROV-1	94	92	295	ND
OVCAR-3	5	58	69	ND
OVCAR-4	9.6	295	540	ND
OVCAR-5	88	40	660	ND
OVCAR-8	15.5	67	230	ND
PXN94	84	43	1450	ND
SKOV-3	46	76	395	58
4IM	>2500	5200	2350	>2500

B. Growth inhibitory properties of geldanamycin, 17AAG, and radicicol against anticancer drug-resistant human tumor cell lines*.

IC_{50} , nM

Cell line	Geldanamycin	17AAG	Radicicol
A2780	7.1	2.4	115
A2780cisR	9.1	1.9	270
4IM	1.3	0.8	2.3
4IMcisR	49	960	325
4IMdoxR	17	1220	88
4IMdoxR (Pgp)	0.35	1.3	0.27
4IMdoxR (Puro)	2500	>2500	565
4IMdoxR (MRP)	51	>2.6	1.7
4IMdoxR (MRP)	97.3	46	280
4IMdoxR (MRP)	337	142	280
4IMdoxR (MRP)	3.5	3.1	1.0

*17AAG = 17-allylamino,17-demethoxygeldanamycin; 17-Amino = 17-amino,17-demethoxygeldanamycin; CRC = Cancer Research Campaign; ICR = Institute of Cancer Research; MRP = multidrug-resistance protein; Pgp = P-glycoprotein; puro = puromycin; ND = not done; RF = resistance factor; cisR = cisplatin-resistant; doxR = doxorubicin-resistant; doxR (Pgp) = doxorubicin-resistant, P-glycoprotein-overexpressing; doxR (MRP) = doxorubicin-resistant, MRP1-overexpressing.

Data are either the mean ± standard deviation (n = 3) or the mean of two determinations.

lines. These lines possess acquired resistance to cisplatin (cisR lines) or to doxorubicin through overexpression of P-glycoprotein (doxR line) or of MRP1 (SKOV-3 S2) (Table 1, B). Although little cross-resistance to geldanamycin was observed in the cisplatin-resistant cell lines, geldanamycin was markedly potent in the P-glycoprotein-overexpressing cell lines and in MRP1-overexpressing cell lines than in the parent lines, suggesting that geldanamycin is a substrate for these multidrug-

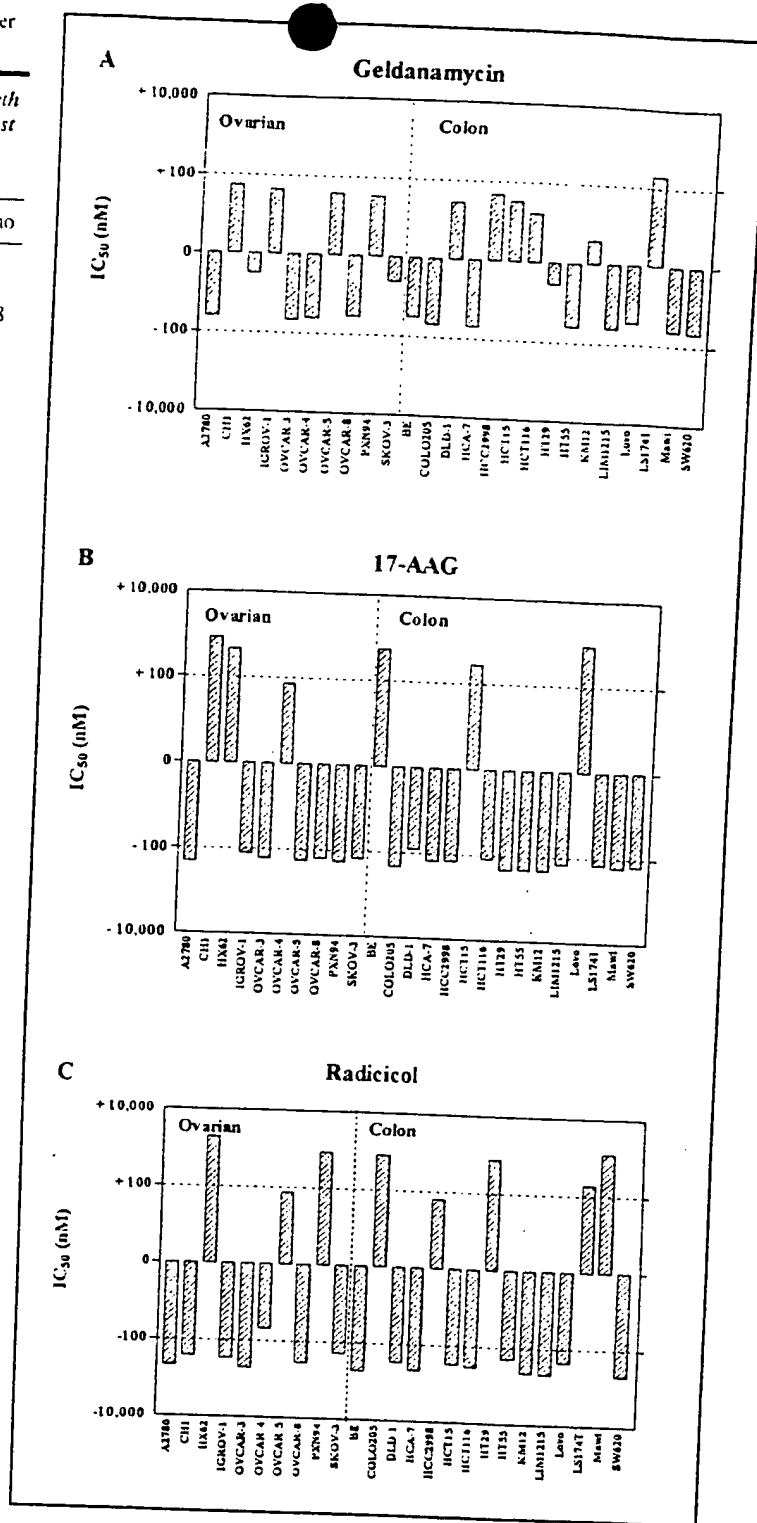


Fig. 2. Patterns of *in vitro* growth inhibition response across 25 human colon and ovarian carcinoma cell lines (as indicated) for geldanamycin (A), 17-allylamino,17-demethoxygeldanamycin (17-AAG) (B), and radicicol (C). Results are displayed as the extent to which the IC_{50} value (mean drug concentrations that inhibit growth by 50% for a 96-hour exposure) for a given cell line was greater or lower than the mean IC_{50} calculated for the entire panel. Values for the mean IC_{50} across the whole panel were as follows: 50.1 nM for geldanamycin, 220.4 nM for 17-AAG, and 587.4 nM for radicicol.

resistant efflux proteins. The picture is rather less clear for 17AAG because the parental CHI ovarian cell line is relatively resistant to 17AAG, although there is at least a 2.5-fold cross-resistance to 17AAG in CHI doxR. The level of cross-resistance

that introduction of the DT-diaphorase gene into BE cells substantially enhanced the potency of streptonigrin, an excellent DT-diaphorase substrate and bioreductive agent. The degree of potentiation correlated with DT-diaphorase levels and activity (117-fold potentiation in BE-F397 clone 5 and 142-fold potentiation in BE-F397 clone 2). Further details will be published elsewhere.

Dose-response curves for geldanamycin and 17AAG in BE vector control cells and BE-F397 clone 2 are shown in Fig. 4, A. Although the two lines showed similar sensitivity to geldanamycin, BE vector control cells lacking DT-diaphorase were markedly less sensitive to 17AAG. The degrees of potentiation (in terms of IC_{50} values) for geldanamycin, 17AAG, 17-amino,17-demethoxygeldanamycin, radicicol, and herbimycin observed when DT-diaphorase was introduced into the BE colon cell line are shown in Fig. 4, B. Notably, a 32-fold potentiation

was observed with 17AAG, whereas a less than threefold potentiation was observed for all other compounds evaluated. In a second test of the effect of DT-diaphorase on the growth inhibitory properties of these compounds (Fig. 4, B), HT29 colon cells (naturally high in DT-diaphorase activity) were compared with BE parent cells (no measurable DT-diaphorase activity). Results generally mirrored those results observed with the isogenic-transfected pair of BE lines, with only 17AAG, of the Hsp90 inhibitors tested, showing a marked DT-diaphorase-mediated differential effect (87-fold potentiation). It is of interest in this pair of lines that HT29 cells had a strikingly greater sensitivity to radicicol than did BE cells, an effect not seen with the isogenic BE cell line pair.

Reduction of 17AAG by Purified Human DT-Diaphorase

Having demonstrated a potentially important role for DT-diaphorase in cellular sensitivity to 17AAG, we used a menadi-one substrate replacement assay as described previously (27,34) to determine the ability of this agent, geldanamycin, and 17-amino,17-demethoxygeldanamycin to act as substrates for purified human DT-diaphorase (Table 2). Streptonigrin (35), an excellent substrate for DT-diaphorase, was also included in the comparison. We found that 17AAG was a reasonable substrate for DT-diaphorase, but it is not appreciably better than geldanamycin or 17-amino,17-demethoxygeldanamycin. This is perhaps surprising in view of the cellular data. The DT-diaphorase-mediated reduction rate was similar for all three analogues, each at a substrate concentration of 10 μM . At 50 μM , 17AAG and 17-amino,17-demethoxygeldanamycin gave twofold to threefold higher rates than geldanamycin, and the difference was even greater at 100 μM . Geldanamycin at 100 μM resulted in substrate inhibition, which was not observed with the other two analogues at 100 μM . The latter two concentrations, however, are much higher than the pharmacologically relevant range. It also should be noted that all three of the ansamycin analogues gave reaction rates that were substantially lower than rates observed for streptonigrin (Table 2). With the structurally distinct Hsp90 inhibitor radicicol, which lacks a quinone moiety, no reduction was observed.

Effects of 17AAG on Hsp90, Hsp70, and Oncogenic Proteins

To determine whether the mode of action of 17AAG was the same in cells expressing low and high levels of DT-diaphorase and to guide the choice of molecular pharmacodynamic markers in the imminent clinical trial, we measured the levels of Raf-1, mutant p53, Hsp90, and Hsp70 proteins in vector control cells and transfected BE cells treated with 17AAG (or geldanamycin). Levels of these proteins 6 and 24 hours after the addition of equitoxic (continuous exposure to 5x and 10x IC_{50}) or equimolar (0.15 and 0.3 μM) geldanamycin or 17AAG are shown in Fig. 5. No change in Hsp90 protein levels was observed. A similar marked reduction, especially at 24 hours, was observed for Raf-1 and p53 proteins in the BE vector control cells and BE-F397 clone 2 cells at equitoxic concentrations. By contrast, an increase in Hsp70 levels was observed. For geldanamycin or 17AAG at equimolar concentrations (0.15 or 0.3 μM), no change in any of the four proteins was observed in the BE vector control cells expressing low levels of DT-diaphorase, consistent with their cellular resistance at these concentrations.

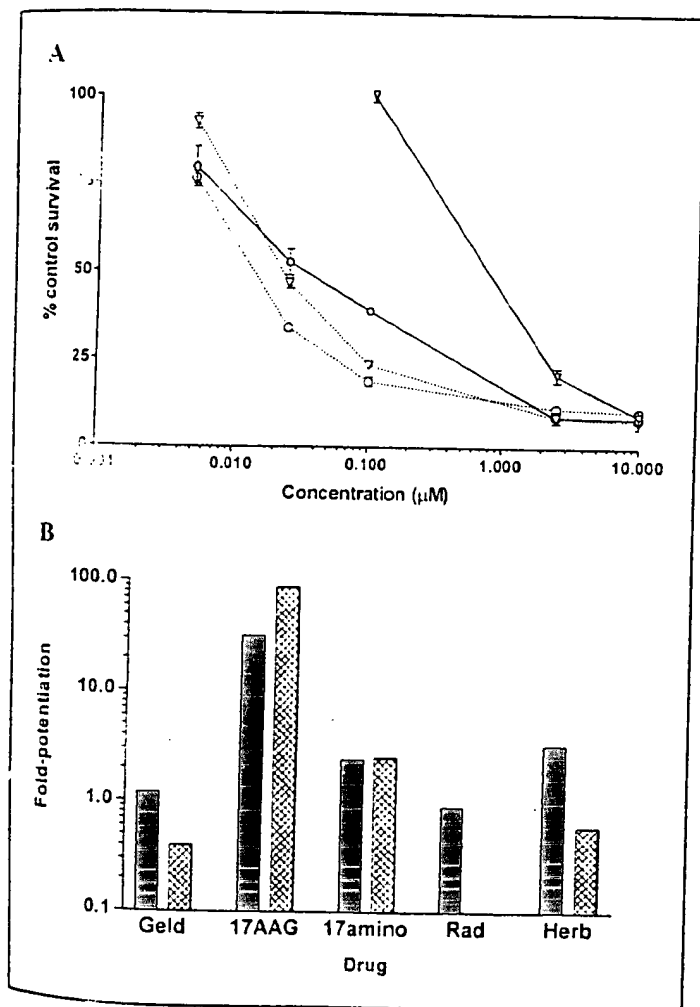


Fig. 4. Effects of DT-diaphorase gene (NQO1) transfection on response to heat shock protein 90 (Hsp90) inhibitors. A) Dose-response curves for BE vector control (V, ▽) and BE-F397 clone 2 (O, C) for geldanamycin (Geld; open symbols, broken lines) and 17AAG (solid symbols, continuous lines). B) Extent of potentiation in high DT-diaphorase-expressing cells compared with low DT-diaphorase-expressing cells for the Hsp90 inhibitors: geldanamycin, 17AAG, 17-amino,17-demethoxygeldanamycin (17-amino), radicicol (Rad), and herbimycin (Herb). Fold-potential = drug concentrations that inhibit growth by 50% (IC_{50}) in cells expressing low levels of DT-diaphorase (BE parental or BE vector control line) / IC_{50} in cells expressing a high level of DT-diaphorase (BE-F397 or HT29 colon line). Solid bars = BE vector control/BE-F397 cells; cross-hatched bars = BE/HT29 cells.

Table 2. Reduction of geldanamycin, 17-allylamino,17-demethoxygeldanamycin (17AAG), and 17-amino,17-demethoxygeldanamycin (17-amino) by purified human DT-diaphorase (at 20 $\mu\text{g/mL}$)*,†

Substrate	Reduction of substrate, μmol of cytochrome <i>c</i> reduced per minute per mg of protein
Menadione, 10 μM	1188.5 \pm 163.7
Streptonigrin, 50 μM	206.1 \pm 6.0
Streptonigrin, 25 μM	159.1 \pm 9.0
Streptonigrin, 10 μM	176.6 \pm 69.5
Geldanamycin, 100 μM	1.5 \pm 0.8
Geldanamycin, 50 μM	7.2 \pm 3.3
Geldanamycin, 25 μM	7.0 \pm 1.0
Geldanamycin, 10 μM	4.3 \pm 0.6
17AAG, 100 μM	20.5 \pm 2.1
17AAG, 50 μM	15.0 \pm 7.9
17AAG, 25 μM	8.3 \pm 1.3
17AAG, 10 μM	3.6 \pm 0.6
17-Amino, 100 μM	17.6 \pm 5.3
17-Amino, 50 μM	22.8 \pm 3.6
17-Amino, 25 μM	11.7 \pm 4.3
17-Amino, 10 μM	6.8 \pm 3.5
Radicicol	ND

*Values are individual or mean \pm standard deviation ($n = 3$).

†ND = not detectable at all concentrations tested.

In Vivo Effects of 17AAG

We determined the effect of 17AAG on the response of the BE vector control cells and BE-F397 cells when grown subcutaneously as solid tumor xenografts in nude mice. 17AAG was administered at the maximum tolerated dose of 80 mg/kg per day intraperitoneally on days 0–4 and days 7–11, a schedule that is active on sensitive xenografts [NCI drug data file on 17AAG and (11)]. The xenograft tumor grown from the transfected BE-F397 cells (Fig. 6, B) was more sensitive than the BE vector control cells (Fig. 6, A). The growth delays, calculated from the time required to reach twice the treatment volume, were 11.4 days for the BE-F397 xenograft and 5.8 days for the vector control. For the HT29 xenograft (and a similar schedule of 80

mg/kg per day intraperitoneally on days 0–3 and days 6–10), a growth delay of 16.6 days was observed (Fig. 6, C). Experiments (not shown) confirmed that the differences in DT-diaphorase expression seen *in vitro* were maintained in the xenograft (data not shown). Thus, the HT29 line (with a naturally high level of DT-diaphorase and also the transfected BE-F397 line) were more sensitive *in vivo* than the BE vector control cells that have a low level of DT-diaphorase activity.

DISCUSSION

17AAG is currently entering phase I clinical trial as the first-in-class Hsp90 inhibitor, under the auspices of the NCI and CRC. Treatment with this drug results in the depletion of a number of important oncogenic proteins, including Raf-1, ErbB2, and mutant p53 proteins, from tumor cells (1,4–6,9). In this article, we show that the levels of DT-diaphorase activity in a tumor cell are an important and statistically significant determinant of how well 17AAG will inhibit the growth of that tumor cell. Evidence for this role of DT-diaphorase comes from the following three observations: 1) There was a statistically significant correlation between DT-diaphorase activity and sensitivity to 17AAG for 11 human colon and ovarian cancer cell lines from the CRC/ICR panel. 2) Subsequent interrogation of data from the NCI panel of 60 human tumor cell lines supported the hypothesis that the level of DT-diaphorase activity was a contributory factor in the differences in the sensitivity of tumor cell lines to 17AAG compared with geldanamycin. [In an analogous way, the differences in sensitivity between methotrexate and trimetrexate in the NCI 60 human tumor cell line panel have been explained by differences in the levels of reduced folate carrier protein (37).] 3) Transfection of DT-diaphorase into the BE human colon cancer cell line, thereby creating pairs of isogenic cell lines differing only in DT-diaphorase expression, resulted in a marked increase in 17AAG-induced growth inhibition *in vitro* and an increased response to 17AAG *in vivo*. The degree

Fig. 5. Representative immunoblots for heat shock protein 90 (Hsp90), RAF-1, p53, and Hsp70 (as indicated) in BE vector control or BE clone 2 cells exposed to equitoxic concentrations (5 \times or 10 \times drug concentrations that inhibit growth by 50% [IC_{50}]) of geldanamycin (0.2 and 0.4 μM for 5 \times and 10 \times IC_{50} in BE vector control cells and 0.1 and 0.2 μM for 5 \times and 10 \times IC_{50} in BE-F397 clone 2 cells, respectively) or 17-allylamino,17-demethoxygeldanamycin (17AAG; 7 and 14 μM for 5 \times and 10 \times IC_{50} in BE vector control cells and 0.15 and 0.3 μM for 5 \times and 10 \times IC_{50} in BE-F397 clone 2 cells, respectively). Two fixed concentrations of 17AAG (0.15 and 0.3 μM) are also shown for RAF-1 in the BE vector control cells. Cells were exposed to drug for 2 hours and harvested 6 and 24 hours after exposure. Lane 1 = 6-hour incubation of untreated cells; lane 2 = 6-hour incubation in geldanamycin (5 \times IC_{50}); lane 3 = 24-hour incubation in geldanamycin (5 \times IC_{50}); lane 4 = 6-hour incubation in geldanamycin (10 \times IC_{50}); lane 5 = 24-hour incubation in geldanamycin (10 \times IC_{50}); lane 6 = 6-hour incubation in 17AAG (5 \times IC_{50}); lane 7 = 24-hour incubation in 17AAG (5 \times IC_{50}); lane 8 = 6-hour incubation in 17AAG (10 \times IC_{50}); lane 9 = 24-hour incubation in 17AAG (10 \times IC_{50}); and lane 10 = 24-hour incubation of untreated cells. Blots for the BE vector control cells and 17AAG are also shown. Lane 11 = 6-hour incubation of untreated cells; lane 12 = 6-hour incubation in 0.15 μM 17AAG; lane 13 = 24-hour incubation in 0.15 μM 17AAG; lane 14 = 6-hour incubation in 0.3 μM 17AAG; lane 15 = 24-hour incubation in 0.3 μM 17AAG; and lane 16 = 24-hour incubation of untreated cells.

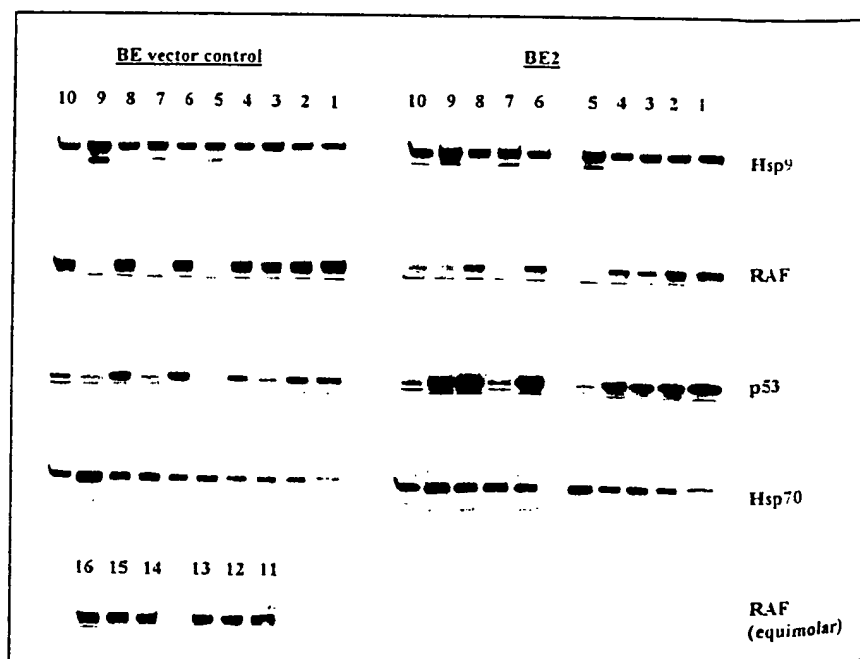


Fig. 6. Xenograft tumor growth of HT29 or BE-F397 tumors in nude mice. Control, 17AAG, and 17AAG + DT-diaphorase.

of *in vitro* expressing the high transfected DT-diaphorase, resulting in a marked increase in 17AAG-induced growth inhibition *in vitro* and an increased response to 17AAG *in vivo*. The degree

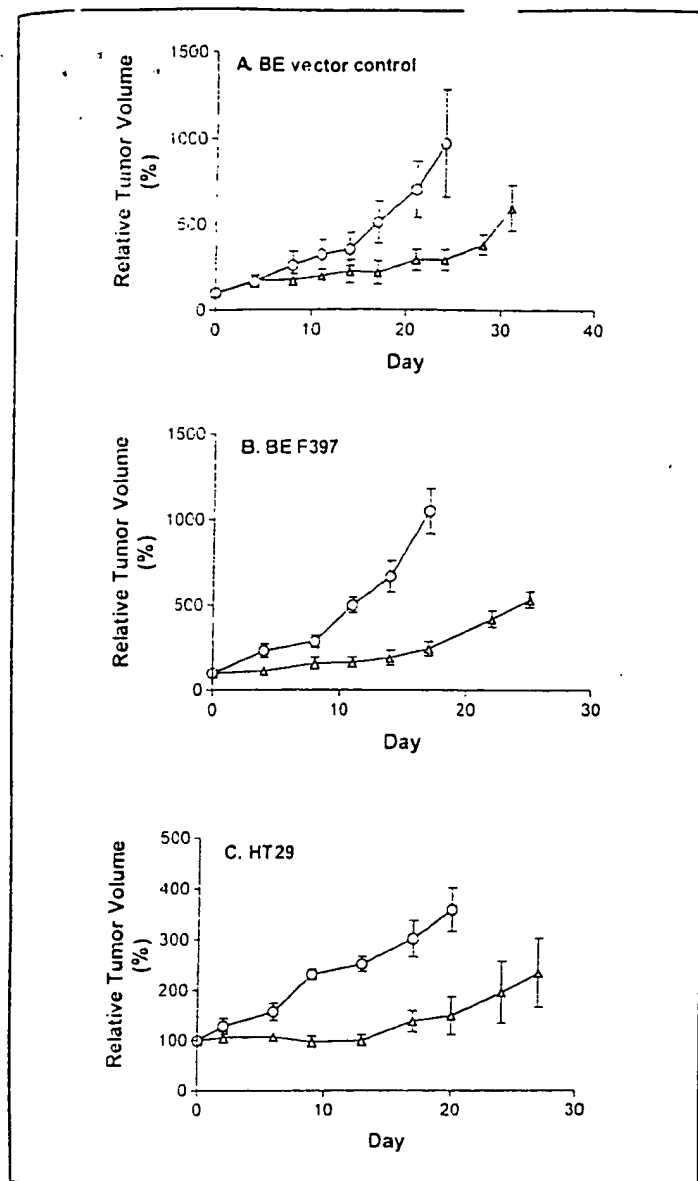


Fig. 6. Effect of DT-diaphorase expression on the response of human tumor xenografts *in vivo* to 17-allylamino,17-demethoxygeldanamycin (17AAG). Tumor growth curves for mice bearing BE vector control (A), BE-F397 clone 2 (B), or HT29 (C) xenografts after treatment with 17AAG. The dosing schedule was 80 mg/kg per day intraperitoneally daily on days 1-4 and days 7-11 for BE tumors and on days 0-3 and days 6-10 for HT29 tumors. Δ = 17AAG; \circ = control. Data for relative tumor volumes are the means (\pm standard deviation) from five animals.

of *in vitro* growth inhibition correlated with the level of enzyme expression, being 32-fold higher in the transfected cell line with the higher levels of DT-diaphorase and 22-fold higher in the transfected cell line with somewhat lower levels. Of interest, the DT-diaphorase effect was not observed with geldanamycin, results in agreement with data from a nonisogenic pair (27). Moreover, the effect was not seen with 17-amino,17-demethoxygeldanamycin, which was identified as the major 17AAG metabolite in human and mouse hepatic preparations (13) and confirmed as such *in vivo* (NCI drug data file on 17AAG). DT-diaphorase activity also appeared unrelated to the potency of radicicol, the structurally distinct Hsp90-binding antibiotic. Indeed, BE cells that express a low level of DT-diaphorase were almost 10-fold more sensitive to radicicol than

were HT29 cells. To express a naturally high level of DT-diaphorase. There was no difference with radicicol in the isogenic transfected BE cell line pair.

The correlation seen between expression of DT-diaphorase activity and sensitivity to 17AAG but not to geldanamycin or radicicol shows that the effect is not generic across all Hsp90 inhibitors or, indeed, across all benzoquinone ansamycins. The precise mechanism by which high levels of DT-diaphorase in tumor cells result in sensitivity to 17AAG is not clear. The observation that DT-diaphorase activity affects tumor cell sensitivity to 17AAG but not to geldanamycin or 17-amino,17-demethoxygeldanamycin is not explicable in terms of their respective behavior as substrates for the purified human enzyme. Although we have demonstrated that 17AAG is a reasonable substrate for human DT-diaphorase, it was not appreciably better than geldanamycin or 17-amino,17-demethoxygeldanamycin, particularly at more relevant drug concentrations. Only at the markedly suprapharmacologic concentrations of 50 and 100 μ M was 17AAG reduced at a statistically significantly faster rate than geldanamycin. For 17-amino,17-demethoxygeldanamycin, there was no appreciable difference in rate compared with geldanamycin.

Given the close structural similarity of 17AAG, 17-amino,17-demethoxygeldanamycin, and geldanamycin (Fig. 1), it is clear that it is the allyl substitution on the amino group at position 17 that is responsible for the DT-diaphorase effect. Preliminary results with a range of 17AAG analogues are consistent with this observation. We hypothesize that the behavior of the reduction product of 17AAG must differ from the reduction products derived from geldanamycin analogues with other substituents.

The xenograft experiment confirmed that DT-diaphorase-transfected BE-F397 cells were more sensitive than BE vector control cells in a solid tumor *in vivo*. The naturally high DT-diaphorase-containing HT29 xenograft was also more sensitive than the BE vector control xenograft. Dose-response data were not generated in these experiments. However, it seems likely that the differences seen in the *in vivo* xenografts were not as large as those observed in the same lines *in vitro*. One factor that would tend to decrease the contribution of DT-diaphorase levels in the xenograft experiments is the metabolism of 17AAG to the 17-amino derivative, which is the major metabolite in the mouse (13). This could be important because we show in this article that sensitivity to the 17-amino metabolite is not affected by DT-diaphorase. Formation of the 17-amino metabolite is catalyzed by cytochrome P450, specifically CYP3A4 in human microsomes (13). Thus, we propose that the sensitivity of a given patient's tumor to 17AAG may be affected by the balance between DT-diaphorase and CYP3A4 metabolism. Consequently, we urge that both enzymes (or surrogates thereof) be monitored in the clinical studies that are now under way with 17 AAG.

We determined that 17AAG was operating through the Hsp90 protein to stimulate degradation of the oncogenic client proteins Raf-1 and mutant p53 by use of 17AAG at equitoxic and equimolar concentrations and cells expressing high and low levels of DT-diaphorase. The depletion of client proteins reported previously for both 17AAG and geldanamycin (4-6,9) was seen in cells expressing high and low levels of DT-diaphorase. At equitoxic concentrations of 17AAG or geldanamycin (5 \times and 10 \times IC₅₀) in the isogenic BE cell lines after 6 hours and, especially, after 24 hours of drug exposure, there was a similar and marked reduction in Raf-1 and mutant p53 proteins. At the fixed

concentrations of 0.15 or 0.3 μ M 17AAG, which inhibited growth of wild-type NQO1-transfected cells but not BE vector control cells, there was no reduction in Raf-1 or p53 protein in cells with low levels of DT-diaphorase, whereas depletion was seen in the cells with high levels of DT-diaphorase that did respond to these concentrations. Thus, target activity was maintained in the presence of the respective active concentrations of 17AAG, independent of the expression of DT-diaphorase. This rules out the possibility that different target mechanisms operate in cells expressing low and high levels of DT-diaphorase. Rather, DT-diaphorase expression increases the potency of 17AAG via client protein depletion.

In contrast to effects reported in melanoma xenografts after administration of 17AAG (11), no difference in the levels of Hsp90 was observed in our experiments. Hsp70 levels, however, were increased, consistent with the removal of Hsp90-induced transcriptional repression of Hsp70 when Hsp90 is inhibited (38). Again, this effect was seen at equitoxic concentrations of 17AAG in both high and low DT-diaphorase lines, consistent with retention of the Hsp90-binding mechanism.

The high constitutive expression of p53 in BE cells suggests a mutant p53 genotype. Effects on mutant p53 were consistent with cell cycle effects of geldanamycin reported in cell lines expressing wild-type or mutant p53 (39). In our own studies on the A2780 human ovarian carcinoma cell line (wild-type for p53) and a subline stably transfected with the viral p53-inactivating gene HPVE6 (40), we found no difference in sensitivity to geldanamycin or 17AAG. Overall, the results indicate that p53 status is unlikely to influence sensitivity to 17AAG.

In summary, although uncertainties remain regarding the precise mechanism involved, our results clearly show that expression of DT-diaphorase can influence a tumor's sensitivity to 17AAG. It is also possible that NQO1 expression could affect toxicity of 17AAG toward normal tissues. There are obvious implications for the clinical evaluation of 17AAG as an anticancer agent because 5%–20% of the population (depending on ethnicity) is homozygous for the genetic polymorphism used in this study, the DT-diaphorase-disabling point mutation in the NQO1 gene present in the BE colon cell line (41). In addition, the expression of DT-diaphorase in human tumors is very variable (25,26), as it is in the cell lines studied herein and elsewhere (22–24). We suggest that, in addition to measuring degradation of oncogenic client proteins and/or an increase in Hsp70 after treatment with 17AAG as potential markers of activity and therapeutic response, NQO1/DT-diaphorase genotype, CYP3A4 status, and also tumor DT-diaphorase levels should be determined. In particular, we propose that these measurements may provide useful indicators of efficacy and/or toxicity and should be considered for the phase I clinical trials of 17AAG that have recently begun under the auspices of the NCI and CRC.

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NOTES

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tion by reducing the surface expression of KDR on HUVECs, or the affinity or total amount of VEGF binding to KDR on HUVECs. Instead, it appears that the durable effect of SU5416 may be due to a residual pool of inhibitor, which is concentrated in cells, that remains associated with cells. The sub-cellular localization and kinetics of elimination of the inhibitor are currently under investigation.

#2843 INHIBITION OF NF- κ B BY A NOVEL PROTEASOME INHIBITOR AND ANTI-TUMOR ACTIVITY IN SQUAMOUS CELL CARCINOMA. J B Sunwoo, Z Chen, G Dong, C V Crowl-Bancroft, N Yeh, J Adams, J Mitchell, E Sausville, and C Van Waes, *Leukosite, Inc, Cambridge, MA, and National Inst of Health, Bethesda, MD*

Squamous cell carcinoma (SCC) of the head and neck has an elevated constitutive activation of the NF- κ B transcriptional regulator. We have evidence suggesting that this activation is important for cell survival, tumor development, and protection from ionizing radiation. Activation of NF- κ B depends on the proteolysis of the inhibitory protein I κ B by the 26S proteasome. In this study, a novel proteasome inhibitor, PS-341 (Leukosite, Inc.), was used to inhibit NF- κ B, and its anti-tumor effects were examined in a variety of murine and human SCC cell lines. A 50% inhibition of NF- κ B was demonstrated by reporter gene and electrophoretic mobility shift assays at 10^{-8} M concentration. This correlated with anti-proliferation assays, demonstrating an IC₅₀ of 10^{-8} M. Flow cytometry was used to show that cytotoxicity was preceded by a cell cycle block at the G2/M transition. Anti-tumor activity was also examined *in vivo*, and a significant dose-dependent response was observed. Because exposure to PS-341 induced a cell cycle block at G2/M and was also found to inhibit induction of NF- κ B by ionizing radiation, we examined the utility of this compound as a sensitizer to ionizing radiation. We found a 30% increase in radiosensitivity by clonogenic assay after accounting for direct cytotoxic effects of the compound. These results suggest that the use of proteasome inhibitors to target the inhibition of NF- κ B may be a useful therapeutic strategy in patients with squamous cell carcinoma of the head and neck.

#2844 RESPONSE OF HUMAN MELANOMAS TO 17-AAG IS ASSOCIATED WITH MODULATION OF THE MOLECULAR CHAPERONE FUNCTION OF HSP90. Angelika Maria Burger, Edward A Sausville, Richard F Camalier, David J Newman, and Heinz H Fiebig, *National Cancer Inst, Bethesda, MD, Tumor Etiology Ctr, Freiburg, Germany, and Univ of Freiburg, Freiburg, Germany*

17-allylaminogeldanamycin (17-AAG, NSC 330507) is a new antitumor agent identified by the NCI which has entered phase I clinical trials in the US. Antitumor activity of geldanamycins has been described to result from degradation of signaling proteins and nuclear hormone receptors by binding their molecular chaperone Hsp90. In this study, two human melanoma xenografts, the 17-AAG sensitive MEXF 276 (T/C = 6%), the resistant MEXF 514 (T/C = 60%), and cell lines derived thereof, were chosen to elucidate 17-AAG effects on its potential target Hsp90 and down-stream effector proteins in a time and concentration dependent manner. Tumor tissues were collected after 48h, 72h, and 10d under 17-AAG treatment (at MTD = 80mg/kg/d, for 2x Qdx5). Cell lines were exposed to drug concentrations which cause total growth inhibition (TGI = 375nM in MEXF 276L, 10 μ M in MEXF 514L cells). By using immunohistochemistry and Western blot analysis we found Hsp90 abundantly expressed in 17-AAG responsive MEXF 276 tumors, but at lower levels in resistant MEXF 514 and in normal tissues. Moreover, whilst 17-AAG treatment did not affect Hsp90 expression in MEXF 514, it caused a rapid decline of Hsp90 in MEXF 276 cells. In latter, this was accompanied by translocation of Hsp90 from cytoplasm and nuclei to cell membranes. In contrast, Hsp72 levels were not changed in either melanoma. As a result of Hsp90 depletion in MEXF 276L cells, down-regulation of Raf-1 and HER-2/neu was observed 8h after drug addition. In MEXF 276 tissues, decrease of Hsp90 was further associated with occurrence of apoptosis. The apoptotic index rose from 9% (48h) over 12% (72h) to 45% (10d) under drug treatment. Our data suggest that the efficacy of 17-AAG is related to its ability to inhibit Hsp90 chaperone function.

#2845 ANTICANCER EFFECTS OF LIPOSOME-ASSOCIATED L AND D STEREOISOMERS OF ET-18- OCH_3 . I. Ahmad, G. R Masters, J. Nguyen, J. Juchupsky, A. S Janoff, and E. Mayhew, *The Liposome Company (TLC), Princeton, NJ*

TLC ELL-12 is a liposome based formulation of ET-18- OCH_3 (1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine), and is currently in Phase I clinical trials. The L isomer of ET-18- OCH_3 is the active ingredient of ELL-12. We have previously shown the therapeutic efficacy of ELL-12 against several experimental mouse tumors. The aim of the present investigation was to determine any difference in toxicity or therapeutic efficacy of ELL-12 when formulated with L or D stereoisomers of ET-18- OCH_3 . The L isomer liposome formulation of ELL-12 significantly reduced toxicity compared to the D isomer liposome formulation when administered once daily, i.v. x 5. L and D isomer formulations of ELL-12 were found to be equally effective in prolonging mean survival time against P388 murine leukemia. However, the L isomer liposome formulation, when administered against established B16/F10 lung tumors, significantly ($p < 0.05$) reduced the mean number of tumor nodules when compared to control or the D isomer liposome formulation. These studies indicate that ELL-12 formulated with the L isomer of ET-18- OCH_3 is less toxic and more effective against B 16/F10 tumor than the D isomer liposomes.

#2846 THE APOPTOTIC EFFECT OF LONG-CHAIN FATTY AMINES ON HUMAN PANCREATIC CANCER CELLS IS MEDIATED BY SIGNALING PATHWAYS INCLUDING MAPK FAMILY AND CASPASES. Mizukami Yusuke, H. Ura, T. Obara, T. Izawa, N. Yanagawa, S. Tanno, Y. Fujimoto, and Y. Kohgo, *Asahikawa Med Coll, Hokkaido, Japan*

Farnesyl transferase inhibitor (FTI) is usually ineffective in Ki-ras transformed cells. However, we have shown that farnesylamine (FA), one of FTI could induce apoptosis in Ki-ras transformed fibroblasts and human pancreatic cancer cell lines (Mol Carcinogenesis, 1998). Therefore, we speculated that FA may have another apoptotic mechanism in addition to the inhibition of farnesylation. Considering the chemical formula of FA, the "long-chain fatty amine (LFA)" structure may have a critical role for this mechanism. In this experiment, we used oleylamine (OA) as LFA and examined the signaling pathways to induce apoptosis in Ki-ras transformed fibroblasts and human pancreatic cancer cell lines. In both cells, apoptosis was induced by OA and JNK activity was increased as well as by FA, but not in parent fibroblast (NIH3T3). Although the OA-induced apoptosis was caspase-dependent, caspase inhibitors did not affect JNK activation. The blockage of JNK activity by dominant negative mutant significantly abrogated the cytotoxic effect of OA and DNA laddering. OA did not act as FTI, but decreased the upregulated ERK activity. In contrast to indispensable effect of JNK in OA-induced apoptosis, attenuated ERK activity alone was not sufficient, but might be required, because MEK inhibitor PD98059 alone did not induce apoptosis. The kinase activity of Akt, which transduce p21 ras mediated survival signaling, resulted in no marked change. Multiple signaling pathways including JNK, ERK, and their downstream caspases mediate the apoptosis and might be shared, at least in part, in FA-induced selective cytotoxicity on Ki-ras mutant cells.

#2847 PHARMACOLOGICAL INDUCTION OF PHOSPHATIDYLINOSITOL ACCUMULATION IS ASSOCIATED WITH CYTOLYSIS OF NEOPLASTIC CELLS. Robert E Finney, E Nudelman, S A Shaffer, T White, S Bursten, L L Leer, N Wang, D Waggoner, J W Singer, and R A Lewis, *Cell Therapeutics, Inc, Seattle, WA*

De novo phospholipid biosynthesis is required for growth of tumor cells. Here, we demonstrate that phospholipid biosynthesis through phosphatidic acid (PA) in neoplastic cells can be exploited for development of cytotoxic anti-cancer agents. PA is a key intermediate for biosynthesis of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) through a diacylglycerol (DAG) intermediate and for biosynthesis of the anionic phospholipids, cardiolipin (CL) and phosphatidylinositol (PI), through a cytidinediphosphate-DAG intermediate. In addition to de novo PA production from lysophosphatidic acid (LPA), production of PA by phospholipase D has been cited among the effects of certain oncogenes (e.g. ras, fps, and src) and growth factors (e.g. PDGF, EGF, FGF, Insulin). CT-2584, a cancer chemotherapeutic drug candidate currently in Phase II clinical trials, decreased utilization of PA for PC biosynthesis and increased PA utilization for PI biosynthesis. A two to three-fold increase in PI was observed in tumor cell lines derived from breast, lung and prostate, was associated with cytotoxic concentrations of CT-2584, and occurred well prior to cytolysis of the tumor cell lines. In contrast, cytotoxic concentrations of cisplatin did not induce accumulation of PI, indicating that PI elevation by CT-2584 was not a general consequence of chemotherapy-induced cell death. Consistent with this mechanism of action, propranolol, an inhibitor of phosphatidic acid phosphohydrolase and PC biosynthesis, was also cytotoxic to tumor cell lines, induced PI accumulation, and was synergistic with CT-2584 in cytotoxicity assays. As expected from the biophysical properties of anionic phospholipids on cellular membranes, CT-2584 cytotoxicity was associated with disruption and swelling of endoplasmic reticulum and mitochondria. We conclude that CT-2584 effects a novel mechanism of action involving modulation of phospholipid metabolism in cancer cells.

#2848 THE EFFECTS OF LYSOPHOSPHATIDYLCHOLINE ON TNF- α PRODUCTION INDUCED BY LIPOSOMAL ET-18- OCH_3 . Marina Y Pushkareva, Andrew S Janoff, and Eric Mayhew, *The Liposome Co, Inc, Princeton, NJ*

The incorporation of 1-o-octadecyl-2-o-methyl-sn-glycero-3-phosphocholine (ET-18- OCH_3) into optimized liposomes (ELL-12) overcomes the non-specific hemolytic effects of ET-18- OCH_3 while maintaining or enhancing anti-cancer efficacy. ELL-12 is currently in Phase I clinical trial. We showed previously that *in vitro* ELL-12 induced growth inhibition is associated with a time- and dose-dependent production of tumor necrosis alpha (TNF- α). As lysophosphatidylcholine (lysoPC) has been shown to modulate the growth inhibiting effects of ELL-12, it was of interest to determine the effects of lysoPC on ELL-12-induced TNF- α production by U-937 cells. We treated U-937 cells with different concentrations of ELL-12 and lysoPC for various times. Maximum of TNF- α production (0.78 ± 0.17 ng per 10^6 cells) was observed after 48 hours of incubation of U-937 cells with 3-4 μ M ELL-12. LysoPC prevented induction of TNF- α production in dose-dependent manner. For example, 20 μ M of lysoPC completely prevented TNF- α production at 48 hours, whereas 2 μ M lysoPC produced 50 % inhibition. The effects on TNF- α production were not directly coupled to the effects of lysoPC on reduction of ELL-12-induced growth inhibition, since 2 μ M lysoPC did not significantly affect ELL-12-induced growth inhibition. ET-18- OCH_3 and lysoPC share structural similarity and have common cellular targets including inhibition of *de novo* phosphatidylcholine synthesis. The possible mechanism of inhibition of ELL-12-induced TNF- α production by lysoPC will be discussed.

(4) - clinical
melanoma

Notes on 17-AAG clinical trials from ASCO meeting, May 2001, San Francisco
L. Fritz

1. Banerji et al (Workman group, UK). (Abstract 326)

Enrolled 21 patients: 6 melanoma (most of any type). 2 patients showed stable disease (both melanoma, at 320 mg/). Drug comes as 25 mg/ml in DMSO.

PD measurements: HSP70 increased
Raf decreased
In one melanoma patient, CDK4 went down and HSP70 went up (no Raf in that tumor)

PK: at 320 mg/ m²: C_{max} = 5-10 uM

Has good xenograft data.

No myelotoxicity even at peak doses.

2. Wilson et al (NIH arm of the study) (Abstract 325)

Richard Wilson presented the poster, but has returned to Northern Ireland. Jean Grem now heads the NIH arm of the clinical trial.

Patients entered:

Colorectal	6
Pancreatic	5
Renal	2
Various	1 each

Total 18 patients

Wilson says Len Neckers was wrong – no lung cancer response was seen – in fact, n9o0 lung cancer patient has been entered at NIH.

They have seen two patients with stable disease: 1 colorectal and 1 renal. But, Wilson says you see these types of patients stabilize spontaneously.

PD measurements:

Had good degradation of Lck and Raf-1 in PBL's (by day 2) also. HSP70 up – all by Western (done by Neckers).

Regarding who at CTEP is in charge of the 17-AAG project:

3

Louise Grachow Runs Investigative Drug Branch in CTEP

Susan Arbuck Runs Developmental Chemotherapy Section (runs it, or is in it?)
Reports to Grachow. Is directly responsible for 17-AAG. Arbuck is the
person we should contact

Dale Shoemaker He was mentioned, but I'm not sure what his role is.

Sherry Ansher Interacts with companies for CTEP. Jean Grem described
her as "more like a lawyer".

3. Munster et al (MSKCC trial data) (Abstract 327)

Pam said she'd send us the slides that comprised her poster

4. Also spoke with the clinician who runs the Mayo Clinic arm of the 17-AAG trial. He said they are having trouble reliably measuring Lck and Raf in their blood samples (Neckers had better gels – but said not all samples had Raf either). NCI seemed to have the best PD measurements of all the sites.